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Note

Quantitative determination of lecithin and sphingomyelin at nanogram levels by high-performance thin-layer chromatography using fluorescence

Preliminary results

L. ZHOU, H. SHANFIELD* and A. ZLATKIS
University of Houston, Chemistry Department, Houston, TX 77004 (U.S.A.)

The use of inorganic acid vapors to induce fluorescence in a variety of compounds has been recently reported by Zhou *et al.*[1] for high-performance thin-layer chromatographic (HPTLC) use. The potentially high sensitivity of the method was noted, but quantitative separations were not carried out. The work reported here deals with the use of HPTLC, together with HNO_3 vapors as part of a fluorescence-inducing procedure, to carry out quantitative analysis of sphingomyelin (S) and lecithin (L) at nanogram levels.

The choice of HNO_3 as the fluorogenic agent was prompted by the observation that its vapors result in the lowest chromatoplate background, while still giving rise to significant fluorescence in the test compounds of many kinds[1].

Interest in sphingomyelin and lecithin was stimulated in part by observations made by Shanfield *et al.*[2] in which fluorescence was readily induced in these compounds by electrical discharge. In addition, there is the well-known lecithin/sphingomyelin (L/S) ratio as a diagnostic tool for assessing infant respiratory distress syndrome, through amniotic fluid analysis[3]. A recent paper by Blass and Ho[4] reported on a fluorescent visualization technique for lecithin and sphingomyelin arising from the incorporation of a fluorescent dye in the solvent system. They established the applicability of their technique for the visualization and quantitation of microgram amounts of lecithin and sphingomyelin. They also concluded that the detection limits of the fluorophores of these compounds were below 0.1 μg. The intensity of fluorescence was observed to decrease slightly over 12 h and quite dramatically over 24 h (without, however, affecting the L/S ratio).

The procedure utilized in the work to be described here, together with HPTLC, is a practical quantitation technique at the level of tens of nanograms. The limit of detection is *ca.* 3 ng for each compound. Finally, the fluorescence is stable for at least 2 weeks (stored in a closed, opaque container).

EXPERIMENTAL

Compounds, solvents, and nitric acid

The sphingomyelin (from bovine brain, Type 1) and lecithin (from egg yolk) employed in these experiments were obtained from Sigma (St. Louis, MO, U.S.A.).

The sphingomyelin was supplied as a solid and the lecithin as a solution (1 g/10 ml of chloroform–methanol (9:1)). Both were stored below 0°C in the course of these experiments. Solutions of each of these compounds were made with ethanol as solvent to provide the range of concentrations investigated. All solvents utilized in this work were of analytical, spectral, or HPLC grade. Nitric acid vapors were derived at room temperature from standard strength HNO_3 (70%) of analytical grade.

Thin-layer chromatoplates

Quantitative calibration data for sphingomyelin and lecithin were obtained on HPTLC silica gel 60 plates (Merck, Darmstadt, G.F.R.). In order to minimize fluorescent background, heating at 260°C for 4 h prior to use was generally sufficient, a procedure which has been previously described[1]. These same HPTLC chromatoplates were used in experiments where sphingomyelin and lecithin were separated chromatographically.

Test procedures

A series of ethanol solutions of sphingomyelin was made ranging in concentration from 50 mg/l to 1 g/l. These stock solutions were then used to spot heat-treated HPTLC chromatoplates with sphingomyelin in amounts from 3 ng to 200 ng. A similar series of lecithin solutions (in ethanol) was prepared (20 mg/l to 660 mg/l) to spot this compound in similar amounts.

Following solvent evaporation of the spotted compounds at room temperature, the chromatoplates were exposed to HNO_3 vapors (also at room temperature) for 10 min. They were then removed and heated in an oven (air) at 180°C for an additional 10 min. The spots were then scanned at 365 nm with a Zeiss KM3 Scanning Densitometer (Carl Zeiss, New York, NY, U.S.A.) without the use of the microoptic accessory. Both peak signal values and peak area values were recorded and plotted graphically against absolute amounts of each compound.

A sphingomyelin–lecithin mixture (100 ng: 52 ng) was spotted on an HPTLC chromatoplate and developed using dichloromethane–ethanol–water (100:25:3) solvent mixture. A continuous development chamber (Regis, Morton Grove, IL, U.S.A.) was utilized for this purpose, and 20 min were required for this separation phase. Subsequently, the plate was subjected to the HNO_3 vapor–air heating cycle previously described, and scanned with the Zeiss instrument.

Excitation spectra were taken of sphingomyelin and lecithin which had been rendered fluorescent by the same procedure. Exciting radiation wavelengths ranged from 260 nm to slightly above 400 nm.

In all the test procedures described, fluorescence was evaluated during the same day of the experiment. However, the measured intensity of fluorescence did not alter noticeably over a period of two weeks of storage in a closed, opaque container.

RESULTS AND DISCUSSION

Quantitative relation between fluorescence and amount

Fig. 1 illustrates the peak signal (mV) *versus* amount of sphingomyelin (365 nm excitation wavelength). The signal increases approximately in linear fashion for sphingomyelin levels from 10 ng to nearly 100 ng. Fig. 2 shows similar data for

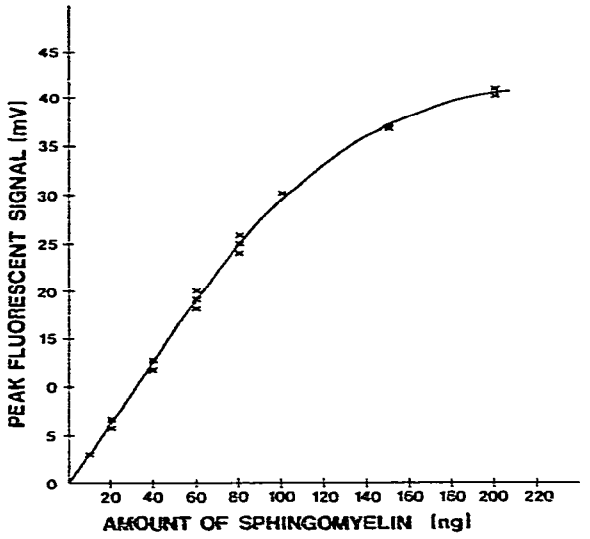

Fig. 1. Quantitative data for sphingomyelin based on peak signal value (mV). Excitation wavelength, 365 nm.

lecithin. Here linearity extends from *ca.* 3 ng to *ca.* 50 ng. Fig. 3 depicts peak area (mV sec) *versus* amount for the same experiments. Here the range of linearity is about the same as for peak signals alone. Thus, for levels of up to a few tens of nanograms, peak signal intensity or peak area appear to be of equal utility from the viewpoint of quantitative linearity. Table I compares a selected L/S ratio to the corresponding ratios of the peak signals obtained at various lecithin (and sphingomyelin) levels. Thus, for the selected ratio of 1.0, the ratio of the corresponding peak signal in-

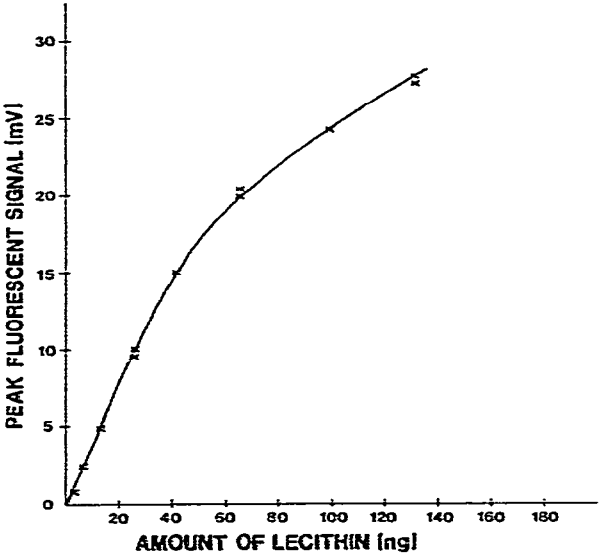

Fig. 2. Quantitative data for lecithin based on peak signal value (mV). Excitation wavelength, 365 nm.

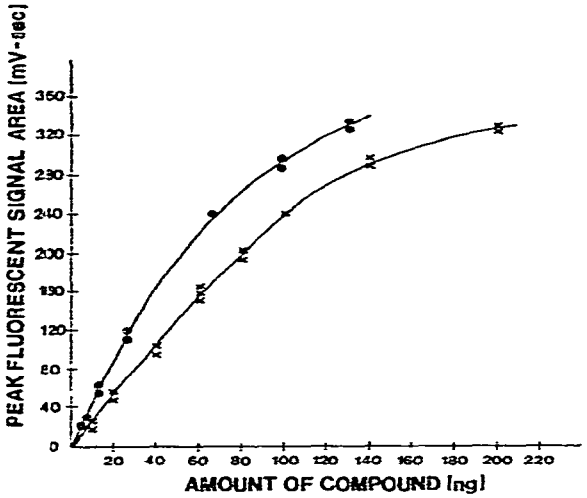

Fig. 3. Quantitative data for lecithin (●) and sphingomyelin (X) based on peak signal area (mV sec). Excitation wavelength, 365 nm.

tensities (taken from the data of Fig. 1) is roughly constant in the linear portion and diminishes slightly in the non-linear regions. Constancy of L/S ratio over a very wide range of L and S amounts would represent an analytical convenience, since the L/S ratio is considered the significant diagnostic factor in antenatal assessment of infant respiratory distress syndrome[3], rather than the absolute levels of lecithin and sphingomyelin.

Chromatographic separation of lecithin and sphingomyelin

Fig. 4 shows the results obtained in scanning (365 nm) an HPTLC chromatoplate on which lecithin and sphingomyelin were separated chromatographically (52 ng lecithin, 100 ng sphingomyelin), using dichloromethane–ethanol–water (100:25:3) solvent system, and rendered fluorescent by the HNO_3 procedure. The L/S ratio obtained from peak heights (0.57) is close to that of the original mixture (0.52). Some impurities appear to be present, adjacent to the sphingomyelin peak.

TABLE I

LECITHIN/SPHINGOMYELIN (L/S) RATIO CALCULATED FROM PEAK FLUORESCENT SIGNAL

Quantitative L/S ratio, 1.0.

Amount of lecithin (= Sphingomyelin) (ng)	*L/S ratio calculated from peak signal values*
20	1.1
40	1.1
60	1.0
80	0.9
100	0.8
120	0.8

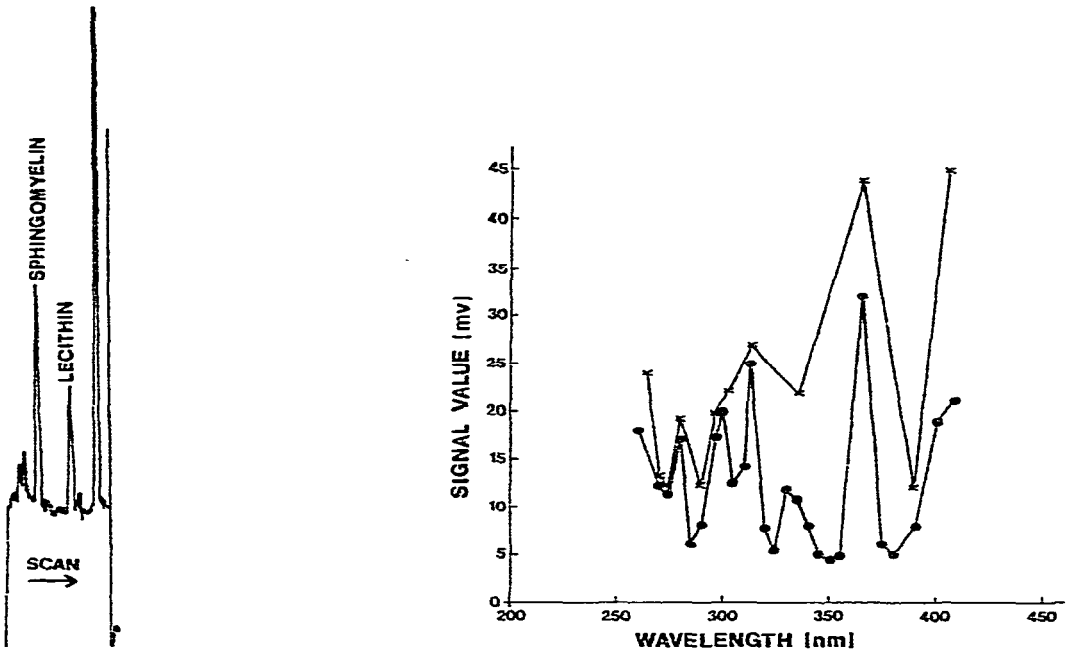

Fig. 4. Results of scanning HPTLC-separated sphingomyelin/lecithin (100 ng/52 ng) after being rendered fluorescent. Scanning wavelength, 365 nm.

Fig. 5. Excitation spectra of fluorescent products of sphingomyelin (X) and lecithin (●).

Excitation spectra of sphingomyelin and lecithin (fluorescent products)

Fig. 5 shows the excitation spectra for the fluorescent products of sphingomyelin and lecithin. For each compound, a distinct strong response occurs near 365 nm, and an additional peak occurs for sphyngomyelin just above 400 nm. Above 414 nm, strong absorption takes place. The peak response at 365 nm for these compounds which have been made fluorescent through the acid vapor treatment, closely duplicates the results obtained by other fluorogenic means previously reported, *e.g.* the NH_4HCO_3–heat cycle reported by Segura and Gotto[5] and the electrical discharge treatment reported by Shanfield *et al.*[2]. This probably reflects a strong commonality of fluorescent chemical structure produced by diverse means.

CONCLUSIONS

We have concluded the following, based on the work described here.

(1) The HNO_3 vapor technique for inducing fluorescence in sphingomyelin and lecithin on HPTLC chromatoplates is a relatively simple and rapid technique, which provides a detection limit of *ca.* 3 ng for each compound.

(2) The fluorescent derivatives of these compounds are stable in intensity for a period of at least two weeks.

(3) A moderately good linear relationship is obtained between either peak fluorescent signal or peak area *versus* amount (up to 100 ng for S and up to 50 ng for

L). The L/S ratio diminishes slightly (compared with the quantitative value) in going from the linear to the non-linear portions of the calibration data.

(4) Excitation spectral data show a peak response at *ca.* 365 nm for each compound, which corresponds closely to results obtained with structurally unrelated compounds, using other fluorescence-inducing procedures.

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